AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Chronic toxicity of *Annona muricata* L. leaf extract

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Article Info

Article history:
Received 28 January 2017
Revised 1 March 2018
Accepted 18 June 2018
Available online 28 February 2019

Keywords:
Annona muricata L.,
Chronic toxicity

Abstract

Annona muricata L., a medicinal plant, has been used for alleviating and curing various illnesses, and in particular, its anticancer activity has made it well-known. The chronic toxicity of the aqueous leaf extract by the oral route was examined in rats. The extract was administered to the five treatment groups at doses of 10 mg/kg/d, 100 mg/kg/d, 1,000 mg/kg/d, 2,000 mg/kg/d and the recovery group for 26 wk compared to the control group that received 10 mL/kg water. The results showed that the lower doses (10 mg/kg/d, 100 mg/kg/d, 1,000 mg/kg/d) did not cause any apparent dose-related changes; however, this should take into consideration that the dose of 2,000 mg/kg/d caused liver abnormality and thrombocytopenia in rats. Therefore, it was concluded that the aqueous extract of *A. muricata* at lower doses did not produce any significant toxicity, but it could cause some abnormalities to rats during the experimental period at the high dose applied.

Introduction

Annona muricata L. known as Soursop or thurian-thet (in Thailand) has been used as a herbal remedy to treat diseases or conditions such as diabetes, inflammation, liver diseases, pain, rheumatism, nervousness, cough, fever, asthma and hypertension (Coria-Téllez et al., 2016). To date, the main phytochemicals found in Soursop leaf have been annonaceous acetogenins which were able to effectively inhibit various cancer cells including lung, breast, colon, pancreas, prostate, and skin cancers (Wu et al., 1995; Kim et al., 1998; Hamizah et al., 2012; Minari and Okeke, 2014). The compounds also effectively reduced blood sugar (Adeyemi et al., 2009) and serum lipid profiles (Adewole and Ojewole, 2009). However, it has been reported that the compounds could cause hepatic and brain disorder (Champy et al., 2004). Acute toxicity of the aqueous leaf extract showed no lethality and signs of toxicity at the dose of 5,000 mg/kg, but the results of histopathology showed fatty degeneration of hepatocytes (Radapong et al., 2016). Annonacin, a major acetogenin in *Annona muricata* could inhibit complex I of the mitochondrial respiratory chain and kill dopaminergic neurons at a potency of 100 times that of 1-methyl-4-phenylpyridinium (Lannuzel et al., 2003). Additionally, the sub-acute toxicity of annonacin evaluated by administering to rats at two different levels of 3.8 mg/kg and 7.6 mg/kg for 28 d confirmed that the neurons deteriorated in the brain basal ganglia and brainstem (Champy et al., 2004). These results were the same symptoms as reported for atypical Parkinsonism gathered from people who consumed fruits or its leaves (infusion and decoction forms) in Guadeloupe, France (Champy et al., 2004). Approximately 70% of acetogenins in the fruit extract was annonacin around 100 times higher than in its aqueous leaf extract (Champy et al., 2005). The fruit pulp of other annonaceous plants containing a high amount of annonacin has also been associated a potential risk of neurodegeneration (Potts et al., 2012). However, the aqueous leaf extraction has not been investigated for its long-term toxicity in animals, particularly chronic toxicity, despite the fact that it is still widely consumed throughout Thailand and tropical countries. The objective of this study was to evaluate the chronic toxicity of the aqueous leaf extract in an animal model using the decoction-extracted compound of the plant collected in local areas and orally administering it to the test animals. These findings may be used as a basis for safety data for prescribing or limiting the usage of this folk medicinal plant.

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https://doi.org/10.34044/j.anres.2019.53.1.09

Materials and Methods

Plant material

Fresh leaves of *Annona muricata* were collected from Phattalung province, Thailand. A voucher specimen (DMSC 5187) was deposited at the Department of Medical Sciences Herbarium, Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand.

Extraction

The fresh leaves were washed with water. All leaves were dried in a ventilated oven at 45–50ºC and then ground into small pieces and refluxed for 1 hr with distilled water (250 g sample powder to 3,000 mL water) and the pellet was re-extracted with fresh solvent twice. All extracted solutions were pooled, filtered with Whatman filter paper no.1 and the drying processes were conducted using rotary evaporation and lyophilization. The residue was determined (yield of 26.19 % dry weight per dry weight). The extract was kept in a light-protected bottle at -20ºC and dissolved in water at different concentrations prior to administration in the test animals.

Quantification of annonacin

Annonacin was investigated using high performance liquid chromatography (HPLC) analysis by applying the conditions from Dang et al (2012). The extract (100 mg) was dissolved in 1 mL methanol, vortexed, then sonicated for 5 min and finally centrifuged to produce the sample solution. A standard annonacin was purchased from Chengdu Biopurify Phytochemicals Ltd., China, and the standard solution was prepared at the concentration of 0.75 mg/mL by dissolving annonacin in methanol. The five additional standard solutions were prepared as concentrations between 0.0056 mg/mL and 0.50 mg/mL. The HPLC analysis was done on a Waters Acquity Ultra Performance LC and separation was achieved on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm in diameter) connected to an Acquity UPLC BEH C18 guard column (20 × 4 mm, 1.7 µm in diameter) at 35°C. The mobile phases were (A) acetonitrile and (B) water using a gradient elution of 35–93% A at 0–4 min, and 93% A at 4–7 min and was pumped at a flow rate of 0.5 mL/min with the injection volume 3 µL. The detection was set at a wavelength of 210 nm.

Animals

A sample of 144 male and female Wistar rats aged around 1 mth and weighing 100 ± 20 g were purchased from the Biolasco Taiwan Co., Ltd., Taipei, Taiwan. The rats were specific-pathogen-free and fed in strict conventional hygienic areas at the Laboratory Animal Center, Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand. All factors were maintained under standard environmental conditions (National Research Council, 2010) which controlled the temperature and relative humidity at 20 ± 2ºC and 50–70 %, respectively and included a 12 hr:12 hr light:darkness cycle. Unlimited feed and clean water were provided to the animals.

Ethics statements

Human and animal care and the biosafety and all experimental procedures were approved by the Institute of Animal Care and Use Committee of the Department of Medical Sciences, Bangkok, Thailand (Approval no. 58–010).

Study of toxicity at 26 weeks

According to the guidelines (World Health Organization, 2000), 72 Wistar rats of each sex were randomly divided into six groups of 12 animals per sex. Group 1 (control group) received water (10 mL/kg/d) for 26 wk. Groups 2–5 were orally treated with the extract suspension at doses of 10 mg/kg/d, 100 mg/kg/d, 1,000 mg/kg/d and 2,000 mg/kg/d. The sixth group treated with 2,000 mg/kg/d was designated as the recovery group. Body weight and food intake were measured weekly and the animals were observed for signs of abnormalities during the 26 wk of treatment. At the end of the treatment period, the first–fifth groups of rats were fasted for 18 hr, then anesthetized with isoflurane and sacrificed by drawing blood samples from the posterior vena cava for hematological and biochemical examinations. The sixth group was further fed without administering the extract suspension for 14 d before being sacrificed to determine whether recovery occurred from the toxic effects observed in the high dose group.

The hematological analysis was performed using an automatic hematological analyzer (Cell Dyn 3500, Abbott). The parameters of the blood samples measure were: hematocrit (Hct), hemoglobin (Hb), red blood cell (RBC), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC), % neutrophil (%N), % lymphocyte (%L), % monocyte (%M), % eosinophil (%E), % basophil (% B) and platelets.

The biochemical analysis of serum samples was performed using an automatic chemistry analyzer (Intergra 400 Plus, Roche). Biochemical parameters measured were: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), total protein, albumin, total bilirubin, creatinine, glucose, uric acid, triglyceride, cholesterol, sodium, potassium and chloride.

The necropsy investigated the positions, shapes, sizes, colors and abnormalities of some visceral organs (brain, heart, kidneys, lungs, trachea, esophagus, stomach, liver, pancreas, intestine, spleen, bladder, salivary glands and adrenal glands). Moreover, the reproductive organs (testes, ovaries and uterus) were also visually observed and recorded for any signs of gross lesions. These organs were then collected, weighed to determine relative organ weights and then preserved in 10% phosphate buffered formalin solution. Tissue slides stained with hematoxylin and eosin were processed. Histopathological examination was performed by a veterinary pathologist.

Statistical analysis

The data were analyzed by analysis of variance (SPSS version 15.0; SPSS Inc., Chicago, IL, USA) followed by the Bonferroni multiple range test to determine significant differences between groups at $p < 0.05$. Histopathological data were evaluated using the the Fisher exact test and the significance level was set at $p < 0.05$.

Results

High performance liquid chromatography analysis

Annonacin was the main acetogenin found in the aqueous extract and indicated that it was the cause of the main toxic effect. Therefore, the amount of the compound was investigated. The standard curve was used to project the correlation between the six concentrations (5.60–750.00 µg/mL) and the area under the peaks produced a high coefficient of determination ($R^2 = 0.9992$). The sample solution was therefore determined and the amount of annonacin was calculated for

the chromatograms shown (Fig. 1). The extract had a concentration of annonacin of 15.60 ± 0.02 µg/ 100 mg sample. This amount of compound in the highest dose (2,000 mg/kg/d) that the rats received in the experiment was around 0.31 mg/kg/d.

Effects of A. muricata extract on body weight, relative food intake and relative organ weight in both male and female animals

There was no difference in the average body weights between extract-treated groups and the control group in both male and female rats throughout the experimental period of 26 wk (Fig. 2). At the beginning, the male rats started at the same average weight as the females. However, in the last week, the male rats weighed approximately 650 g and were twice as heavy as the females. The relative food consumption of animals receiving the extract was significantly different from the control groups for several weeks. Male rats receiving 2,000 mg/kg/d of the extract (Group 5) had significantly lower relative food intake than the control group in weeks 20–23 and in week 25, while the other treated groups were not significantly different from the control group of females (Fig. 3). The relative organ weight of male rats was not significantly different between the treated and control groups (Table 1). Nevertheless, there were differences in some organs (heart, lung, liver and right adrenal gland of the female animals treated at the dose of 2,000 mg/kg/d had higher weight than the control). Moreover, the kidneys from the female rats treated at doses of 1,000 and 2,000 mg/kg/d were also significantly higher than the control (Table 2).

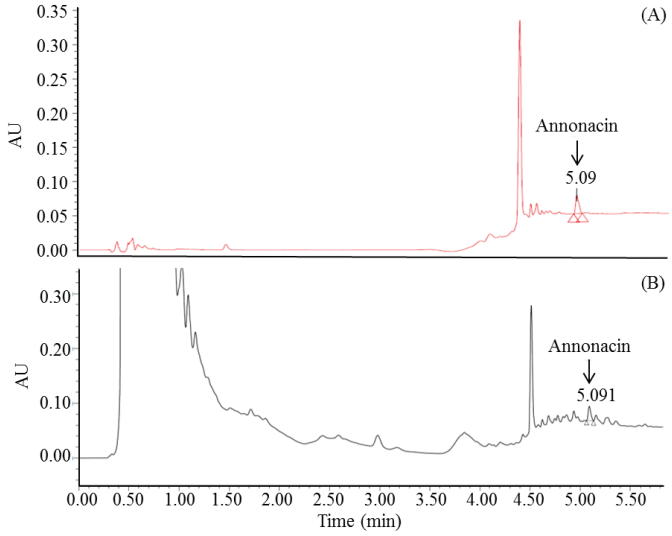

Fig. 1 Chromatograms of: (A) standard annonacin (22.70 µg/mL); (B) aqueous leaf extract of *A. muricata* (100.00 mg/mL)

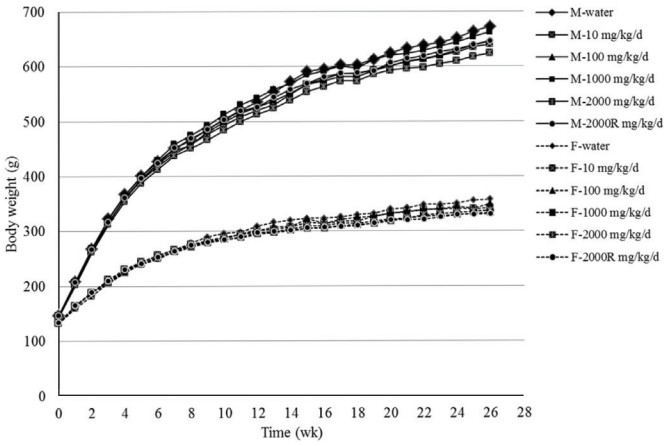

Fig. 2 Growth curves of male (M) and female (F) rats receiving *A. muricata* for 26 wk

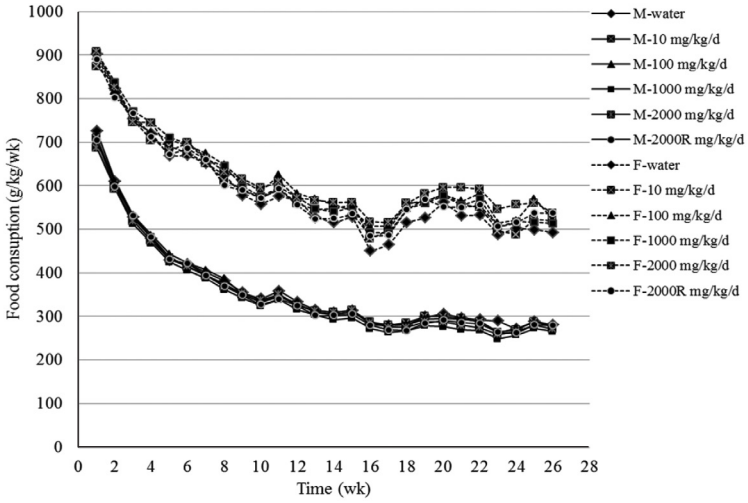

Fig. 3 Relative food consumption of male (M) and female (F) rats receiving *A. muricata* for 26 wk

Table 1 Relative organ weight (g/kg of body weight) and body weight (g) of male rats (mean ± SD) receiving *A. muricata* for 26 wk

Organ	Dose of *A. muricata* (mg/kg/d)					
	DW	10	100	1,000	2,000	2,000–R
	n = 10	n = 12	n= 12	n = 12	n = 12	n = 12
Brain	3.54 ± 0.32	3.54 ± 0.26	3.51 ± 0.27	3.39 ± 0.44	3.63 ± 0.34	3.57 ± 0.45
Heart	2.48 ± 0.16	2.50 ± 0.21	2.51 ± 0.21	2.38 ± 0.25	2.64 ± 0.22	2.59 ± 0.26
Lung	2.81 ± 0.33	2.85 ± 0.29	2.79 ± 0.24	2.65 ± 0.32	2.95 ± 0.25	2.98 ± 0.36
Liver	24.54 ± 2.70	23.07 ± 2.48	22.79 ± 1.32	24.29 ± 1.49	26.39 ± 1.70	25.28 ± 2.85
Stomach	4.05 ± 0.42	3.82 ± 0.40	3.83 ± 0.28	3.78 ± 0.45	4.00 ± 0.34	4.09 ± 0.48
Spleen	1.64 ± 0.17	1.47 ± 0.23	1.48 ± 0.11	1.53 ± 0.19	1.53 ± 0.19	1.61 ± 0.22
Right kidney	2.79 ± 0.28	2.74 ± 0.21	2.66 ± 0.18	2.67 ± 0.18	2.82 ± 0.16	3.00 ± 0.29
Left kidney	2.77 ± 0.28	2.65 ± 0.28	2.58 ± 0.19	2.64 ± 0.14	2.74 ± 0.16	2.87 ± 0.22
Right testis	4.43 ± 0.40	4.69 ± 0.50	4.34 ± 0.71	4.48 ± 0.57	4.85 ± 0.49	4.61 ± 0.71
Left testis	4.47 ± 0.42	4.76 ± 0.46	4.36 ± 0.65	4.66 ± 0.96	4.73 ± 0.55	4.58 ± 0.75
Right adrenal	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Left adrenal	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.10 ± 0.16	0.05 ± 0.01
Bladder	0.30 ± 0.03	0.29 ± 0.03	0.28 ± 0.04	0.29 ± 0.04	0.28 ± 0.06	0.28 ± 0.04
Initial body weight	147.07 ± 6.07	146.62 ± 6.50	145.65 ± 4.52	148.05 ± 6.24	145.10 ± 6.11	146.56 ± 5.64
Final body weight	648.87 ± 61.67	618.64 ± 45.44	624.61 ± 41.57	641.07 ± 69.06	602.75 ± 29.66	632.68 ± 55.40

DW = distilled water; R = recovery group

Table 2 Relative organ weight (g/kg body weight) and body weight (g) of female rats (mean ± SD) receiving *A. muricata* for 26 wk

Organ	Dose of *A. muricata* (mg/kg/d)					
	DW	10	100	1,000	2,000	2,000–R
	n = 12	n = 12	n = 11	n = 12	n = 12	n = 12
Brain	6.00 ± 0.63	6.14 ± 0.42	6.18 ± 0.62	6.31 ± 0.47	6.63 ± 0.78	6.59 ± 0.75
Heart	2.99 ± 0.24	3.12 ± 0.31	3.07 ± 0.30	3.27 ± 0.28	3.42 ± 0.22*	3.33 ± 0.33*
Lung	3.80 ± 0.38	4.04 ± 0.51	4.05 ± 0.41	4.29 ± 0.45	4.32 ± 0.53*	4.39 ± 0.54*
Liver	22.17 ± 1.42	23.65 ± 2.59	23.00 ± 1.98	24.36 ± 2.71	25.32 ± 1.94*	24.06 ± 1.95
Stomach	4.69 ± 0.71	5.11 ± 0.88	4.91 ± 0.50	5.09 ± 0.84	5.17 ± 0.40	5.33 ± 0.41
Spleen	1.85 ± 0.11	1.99 ± 0.23	2.00 ± 0.33	2.07 ± 0.29	1.94 ± 0.18	2.15 ± 0.43
Right kidney	2.71 ± 0.27	2.92 ± 0.26	2.78 ± 0.21	3.13 ± 0.24*	3.19 ± 0.27*	3.08 ± 0.32*
Left kidney	2.72 ± 0.31	2.82 ± 0.26	2.80 ± 0.25	3.05 ± 0.22*	3.12 ± 0.26*	3.00 ± 0.28*
Right adrenal	0.12 ± 0.03	0.12 ± 0.01	0.12 ± 0.02	0.14 ± 0.02	0.14 ± 0.02	0.15 ± 0.03*
Left adrenal	0.13 ± 0.03	0.13 ± 0.04	0.13 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.04
Bladder	0.34 ± 0.09	0.35 ± 0.05	0.38 ± 0.08	0.37 ± 0.05	0.38 ± 0.06	0.40 ± 0.07
Uterus	2.36 ± 0.86	2.73 ± 0.96	3.00 ± 0.81	3.22 ± 1.18	3.05 ± 1.16	2.57 ± 0.93
Right ovary	0.22 ± 0.06	0.24 ± 0.04	0.26 ± 0.05	0.26 ± 0.04	0.25 ± 0.07	0.27 ± 0.05
Left ovary	0.24 ± 0.07	0.24 ± 0.05	0.27 ± 0.05	0.27 ± 0.05	0.24 ± 0.08	0.28 ± 0.05
Initial body weight	132.78 ± 7.13	132.80 ± 5.98	133.87 ± 7.16	133.54 ± 7.68	133.83 ± 6.17	133.76 ± 4.71
Final body weight	340.57 ± 39.35	327.18 ± 25.28	332.96 ± 40.05	317.12 ± 13.72	320.37 ± 27.20	314.11 ± 25.96

DW = distilled water**;** R = recovery group

* significantly different from the control group ($p < 0.05$)

Effect of A. muricata extract on hematological parameters

There were no differences in the values of % hematocrit, RBC, hemoglobin, MCV, MCH, MCH, WBC, %N, %E, %L, %M and %B and the platelets between the extract-treated groups and the control group of male rats. While the numbers of platelets of the female rats treated at the dose of 2,000 mg/kg/d were significantly lower than the control, the change was still within the normal range (Gikinis and Clifford, 2008) as shown in Tables 3 and 4.

Effect of A. muricata extract on blood chemistry

In the male and female rats, no differences were found in the serum levels of ALP, ALT, AST, BUN, creatinine, total protein, albumin, total bilirubin, glucose, uric acid, triglyceride, cholesterol between the extract-treated groups and the control groups (Tables 5 and 6).

Table 3 Hematological values of male rats (mean ± SD) receiving *A. muricata* for 26 wk

Parameter	Dose of *A. muricata* administered (mg/kg/d)						Reference range††
	DW	10	100	1,000	2,000	2,000–R	
	n = 10	n = 12	n = 12	n = 12	n = 12	n = 12	
Hematocrit (%)	44.16 ± 1.11	45.13 ± 1.07	44.34 ± 2.24	43.73 ± 1.88	44.03 ± 1.97	44.43 ± 1.55	38.5–52.0
RBC ($\times 10^6$cells/mm^3)	7.74 ± 0.23	7.92 ± 0.19	7.80 ± 0.32	7.81 ± 0.40	7.67 ± 0.39	7.79 ± 0.28	7.62–9.99
Hemoglobin (g/dL)	14.10 ± 0.32	14.28 ± 0.31	14.23 ± 0.69	14.12 ± 0.54	14.18 ± 0.57	13.98 ± 0.45	13.6–17.4
MCV (µm^3/red cell)	57.05 ± 1.60	56.96 ± 1.04	56.84 ± 1.51	56.08 ± 2.35	57.45 ± 2.38	57.03 ± 1.91	46.3–56.2
MCH (pg/red cell)	18.22 ± 0.46	18.03 ± 0.42	18.25 ± 0.44	18.12 ± 0.77	18.50 ± 0.60	17.97 ± 0.56	16.3–19.5
MCHC (g/dL RBC)	31.94 ± 0.54	31.66 ± 0.48	32.11 ± 0.42	32.30 ± 0.46	32.19 ± 0.52	31.49 ± 0.41	31.9–38.5
WBC ($\times 10^3$cells/mm^3)	7.21 ± 1.66	7.18 ± 1.67	7.05 ± 1.73	6.91 ± 1.37	6.64 ± 1.41	7.65 ± 1.52	1.98–11.06
Neutrophil (%)	28.45 ± 6.24	31.35 ± 7.20	29.40 ± 6.70	32.09 ± 7.71	30.53 ± 5.63	29.09 ± 5.69	9.0–49.3
Eosinophil (%)	3.61 ± 0.62	3.48 ± 1.06	3.77 ± 0.86	3.55 ± 0.59	3.42 ± 0.74	3.49 ± 1.06	0.4–4.0
Lymphocyte (%)	54.96 ± 6.78	51.69 ± 7.36	54.24 ± 7.05	52.00 ± 8.46	52.18 ± 8.02	54.20 ± 9.51	44.7–87.1
Monocyte (%)	7.26 ± 2.53	8.35 ± 3.31	8.59 ± 2.36	8.39 ± 2.72	9.65 ± 2.30	8.41 ± 3.12	1.0–3.6
Basophil (%)	5.70 ± 3.04	5.12 ± 2.37	3.99 ± 0.83	3.97 ± 2.29	4.21 ± 1.55	4.81 ± 2.56	0–0.6
Platelet ($\times 10^3$cells/mm^3)	942.40 ± 104.00	965.29 ± 81.40	969.13 ± 67.03	1038.79 ± 104.99	934.92 ± 71.30	1,015.21 ± 72.73	574 –1,253

DW = Distilled water; †R = Recovery group; †† Mary et al. (2008)

Table 4 Hematological values of female rats (mean ± SD) receiving *A. muricata* for 26 wk

Parameter	Dose of *A. muricata* administered (mg/kg/d)						Reference range††
	DW	10	100	1,000	2,000	2,000–R	
	n = 12	n = 12	n = 11	n = 12	n = 12	n = 12	
Hematocrit (%)	41.68 ± 2.01	42.62 ± 1.69	43.76 ± 1.63	42.58 ± 1.78	42.88 ± 1.78	43.49 ± 1.93	38.5–49.2
RBC (1×10^6 cells/mm^3)	6.98 ± 0.31	7.20 ± 0.31	7.34 ± 0.31	7.09 ± 0.26	7.15 ± 0.27	7.31 ± 0.38	7.16–9.24
Hemoglobin (g/dL)	13.68 ± 0.43	13.88 ± 0.37	14.04 ± 0.34	13.76 ± 0.45	13.85 ± 0.48	13.98 ± 0.63	13.7–17.2
MCV (µm^3/red cell)	59.71 ± 1.74	59.21 ± 1.75	59.66 ± 2.17	60.04 ± 1.36	59.97 ± 1.33	59.51 ± 2.46	50.3–57.0
MCH (pg/red cell)	19.60 ± 0.58	19.28 ± 0.62	19.15 ± 0.78	19.42 ± 0.41	19.38 ± 0.32	19.14 ± 0.76	17.6–20.3
MCHC (g/dL RBC)	32.84 ± 0.96	32.59 ± 0.70	32.10 ± 0.69	32.34 ± 0.49	32.33 ± 0.74	32.18 ± 0.19	33.2–37.8
WBC (1×10^3 cells/mm^3)	3.50 ± 1.04	3.45 ± 1.06	3.75 ± 0.79	3.62 ± 1.01	3.83 ± 0.67	3.19 ± 1.25	0.96–7.88
Neutrophil (%)	31.82 ± 9.37	27.25 ± 9.03	25.82 ± 6.88	26.89 ± 4.67	28.44 ± 8.49	24.26 ± 6.36	8.8–43.8
Eosinophil (%)	8.32 ± 5.32	5.86 ± 3.18	5.41 ± 2.80	4.77 ± 2.70	5.46 ± 2.61	5.15 ± 1.49	0.3–4.7
Lymphocyte (%)	47.45 ± 12.07	54.70 ± 10.36	54.35 ± 7.76	52.78 ± 8.13	54.06 ± 9.20	55.94 ± 8.78	48.9–88.1
Monocyte (%)	7.58 ± 3.04	7.52 ± 2.06	8.35 ± 3.09	9.35 ± 3.07	7.74 ± 2.07	9.17 ± 3.85	1.0–3.6
Basophil (%)	4.85 ± 2.34	4.67 ± 1.76	6.08 ± 3.37	6.22 ± 2.69	4.32 ± 1.03	5.47 ± 2.15	0–0.7
Platelet (1×10^3 cells/mm^3)	938.63 ± 107.7	896.33 ± 102.10	828.55 ± 89.51	850.08 ± 86.14	816.75 ± 89.25*	780.92 ± 51.80*	599–1,144

DW = distilled water; R = recovery group; RBC = Red blood cell

†† Mary et al. (2008)

* significant difference from the control group ($p < 0.05$)

Table 5 Biochemical values of male rats (mean ± SD) receiving *A. muricata* for 26 wk

Parameter	Dose of *A. muricata* administered (mg/kg/d)						Reference range††
	DW	10	100	1,000	2,000	2,000–R	
	n = 10	n = 12	n = 12	n = 12	n = 12	n = 12	
ALP (U/L)	42.30 ± 9.49	44.33 ± 18.48	45.17 ± 12.71	39.50 ± 9.51	46.17 ± 16.20	37.08 ± 6.17	36–131
ALT (U/L)	28.5 ± 2.07	29.67 ± 8.55	30.67 ± 9.43	24.42 ± 2.78	29.90 ± 10.07	27.33 ± 3.73	19–48
AST (U/L)	84.5 ± 16.78	85.92 ± 17.42	83.92 ± 16.90	74.25 ± 11.27	90.25 ± 17.09	87.08 ± 8.25	63–175
BUN (mg/dL)	13.78 ± 0.92	13.76 ± 1.25	13.61 ± 1.89	13.06 ± 1.50	13.90 ± 1.47	13.08 ± 1.69	10.7–20.0
Creatinine (mg/dL)	0.30 ± 0.03	0.30 ± 0.04	0.27 ± 0.10	0.27 ± 0.04	0.26 ± 0.09	0.27 ± 0.04	0.30–0.50
Total protein (g/dL)	5.43 ± 0.29	5.44 ± 0.19	5.46 ± 0.21	5.49 ± 0.21	5.34 ± 0.23	5.33 ± 0.21	5.6–7.6
Albumin (g/dL)	3.52 ± 0.35	3.56 ± 0.21	3.73 ± 0.11	3.60 ± 0.25	3.66 ± 0.16	3.49 ± 0.20	3.6–4.7
Total bilirubin(mg/dL)	0.03 ± 0.03	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.04 ± 0.01	0.04–0.20
Glucose (mg/dL)	162.64 ± 21.15	163.22 ± 13.36	169.54 ± 13.91	165.55 ± 10.49	165.04 ± 17.65	160.11 ± 20.67	106–184
Uric acid (mg/dL)	1.19 ± 0.35	0.99 ± 0.14	0.95 ± 0.16	0.96 ± 0.12	0.98 ± 0.11	0.93 ± 0.15	–
Triglyceride (mg/dL)	97.54 ± 32.25	86.11 ± 19.50	74.21 ± 13.39	112.36 ± 56.61	91.32 ± 33.49	98.52 ± 48.29	27–160
Cholesterol (mg/dL)	96.58 ± 36.89	88.21 ± 27.82	81.65 ± 14.02	91.45 ± 33.55	73.28 ± 18.38	113.31 ± 48.61	37–95
Sodium (mmol/L)	124.60 ± 2.63	124.83 ± 2.41	125.75 ± 2.60	124.08 ± 3.09	124.67 ± 2.57	123.83 ± 1.19	137–147
Potassium (mmol/L)	4.94 ± 0.40	5.16 ± 0.44	5.02 ± 0.41	5.03 ± 0.32	4.93 ± 0.26	4.81 ± 0.26	3.88–6.11
Chloride (mmol/L)	102.10 ± 0.88	102.00 ± 1.04	102.08 ± 1.31	102.08 ± 1.31	102.42 ± 1.16	101.83 ± 1.53	98–106

DW = distilled water; R = recovery group

†† Mary et al. (2008)

Table 6 Biochemical values of female rats (mean ± SD) receiving *A. muricata* for 26 wk

Parameter	Dose of *A. muricata* administered (mg/kg/d)						Reference range††
	DW	10	100	1,000	2,000	2,000–R	
	n = 12	n = 12	n = 11	n = 12	n = 12	n = 12	
ALP (U/L)	20.75 ± 5.15	20.42 ± 4.96	20.82 ± 4.69	23.75 ± 8.19	20.50 ± 4.30	18.92 ± 3.73	18–62
ALT (U/L)	21.92 ± 2.91	23.25 ± 7.30	19.91 ± 2.84	22.50 ± 5.37	21.50 ± 3.68	22.08 ± 6.86	14–64
AST (U/L)	79.67 ± 10.17	83.42 ± 11.29	86.36 ± 17.88	83.33 ± 14.43	95.67 ± 11.58	87.92 ± 24.77	64–222
BUN (mg/dL)	17.68 ± 2.65	19.09 ± 3.51	18.82 ± 3.29	19.53 ± 3.94	18.12 ± 3.20	17.34 ± 2.31	11.7–25
Creatinine (mg/dL)	0.37 ± 0.03	0.34 ± 0.04	0.34 ± 0.04	0.39 ± 0.09	0.34 ± 0.06	0.36 ± 0.06	0.3–0.6
Total protein (g/dL)	5.69 ± 0.20	5.67 ± 0.30	5.60 ± 0.35	5.70 ± 0.38	5.65 ± 0.38	5.33 ± 0.27	5.7–8.3
Albumin (g/dL)	4.43 ± 0.24	4.28 ± 0.21	4.20 ± 0.28	4.29 ± 0.31	4.31 ± 0.25	4.18 ± 0.30	3.7–5.8
Total bilirubin(mg/dL)	0.08 ± 0.04	0.06 ± 0.04	0.08 ± 0.03	0.09 ± 0.05	0.08 ± 0.03	0.08 ± 0.04	0.07–0.21
Glucose (mg/dL)	142.70 ± 15.19	144.12 ± 12.25	135.00 ± 12.38	145.93 ± 12.52	141.73 ± 16.68	141.91 ± 18.26	89–163
Uric acid (mg/dL)	0.97 ± 0.23	0.81 ± 0.14	0.85 ± 0.13	0.93 ± 0.12	0.79 ± 0.12	0.77 ± 0.21	–
Triglyceride(mg/dL)	61.97 ± 12.72	53.37 ± 15.76	53.74 ± 15.15	56.01 ± 12.33	53.68 ± 14.27	51.68 ± 14.82	16–175
Cholesterol (mg/dL)	55.91 ± 10.00	62.32 ± 16.56	51.49 ± 11.64	62.95 ± 20.83	58.99 ± 15.35	55.97 ± 25.48	23–97
Sodium (mmol/L)	124.58 ± 1.73	123.33 ± 1.23	123.82 ± 2.60	124.33 ± 1.92	123.25 ± 1.36	122.92 ± 1.38	135–146
Potassium (mmol/L)	4.31 ± 0.42	4.28 ± 0.29	4.23 ± 0.11	4.13 ± 0.17	4.02 ± 0.30	4.00 ± 0.19	3.37–5.11
Chloride (mmol/L)	103.58 ± 1.16	103.50 ± 1.38	103.82 ± 0.87	103.42 ± 0.90	102.75 ± 1.36	103.25 ± 1.06	97–106

DW = distilled water; R = recovery group

†† Mary et al. (2008)

Effect of A. muricata extract on histopathology of internal organs

During the histopathological examination of the organs, some liver lesions were found. The liver showed fatty changes in all groups of male rats and some high doses (1,000 mg/kg/d and 2,000 mg/kg/d) in females including vacuolar degeneration, but these were not significantly different from the control group. (Table 7).

Discussion

The aqueous leaf extract of *A. muricata* used in the experiment was quantified for the amount of annonacin that has been reported to be the main acetogenin and cause of neurodegeneration in rats (Champy et al., 2004). The annonacin was approximately 156 mg/kg dried extract, which was about three times higher than reported by Champy et al. (2004) though its pulp was reported to produce a higher amount of 525 mg/ kg dried extract (Champy et al., 2005). The given doses of the crude leaf extract were 2,000 mg/kg/d, 1,000 mg/kg/d, 100 mg/kg/d and 10 mg/kg/d were approximately equal to the annonacin amounts of 0.31 mg/kg/d, 0.16 mg/kg/d, 0.02 mg/kg/d and 0.002 mg/kg/d, respectively.

The plant extract had significant effects on the food intake of the male rats treated with 2,000 mg/kg/d in some weeks in the last quarter of the experimental period, which could be observed in the growth curves and relative food consumption graph (Figs. 2 and 3), although there was no interference with their weight gain. The gaining of organ weight in the female rats such as in the liver, heart, kidneys or adrenal glands might have been the effect of organ hypertrophy by an increase in weight of the metabolism organs, which resulted from receiving high doses of the extract. These changes could be considered as a normal adaptive response to a xenobiotic and have little relevance to humans (Hall et al., 2012). The histopathology showed some lesions of fatty degeneration or hepatic steatosis. Most prevalences were mild effects based on the scoring of severity applied from Petta et al. (2015). The changes were not significantly different from the control and there was no correlation between biochemical parameters such as ALP, ALT, AST, albumin or bilirubin and the incidences. The lesion could be reversible with the absence of the treated extract for 2 wk as indicated by the results of the recovery group. The significantly decrease in platelets in the female rats treated with the extract at the dose of 2,000 mg/kg/d might have been the result of chronic disorder of the liver. This change also occurred in the male rats, but it was not significant. Recently, it has been proved the thrombocytopenia was the most common hematological abnormality found in patients with chronic liver disease (Mitchell et al., 2016).

The extract of *A. muricata* seemed not to cause apparent toxicity at the given doses based on the experimental evidence. A previous sub-acute toxicity study (28 d) reported that the *A. muricata* leaf extract (mixed with other plants) at the administered dose of 2,000 mg/ kg/d showed no toxicity in animals and no genotoxic activity (Semple et al., 2016). Another sub-acute toxicity study found that the water extract had hypoglycaemic and hypolipidaemic activities at low doses and could cause kidney disorders at the dose of 2,500 mg/kg/d (Arthur et al., 2011). Nevertheless, according to Champy et al. (2004), the rodents treated with the high dose of annonacin up to 7.6 mg/kg/d intravenously had neurotoxicity, but, interestingly, there were no changes including body weight, food intake or general behavior in the treated animals, and no signs of systemic illness; additionally, there was no systemic disorder found in the histopathological examination of any visceral organs.

Overall, the extract at the lower doses showed no apparent signs of chronic toxicity during the experimental period, perhaps because the extract did not cause adverse effects to any specifically targeted organs. Although annonacin has been investigated for causing neurodegeneration for more than a decade (Lannuzel et al., 2003; Champy et al., 2004; Champy et al., 2005; Potts et al., 2012), it was found that a very small amount of annonacin administered by oral route was detected in the brain (1.4 ng/g for 10 mg/kg at 48 hr) by Bonneau et al. (2016). This indicated that it was very weakly distributed and could cross the blood-brain barrier. In the current experiment, the amount of annonacin in the extract at the highest dose (2,000 mg/kg/d) was over 30 times lower than the level in Bonneau et al. (2016). While there were no apparent lesions in the brain specimens from the current study, the possibility cannot be ruled out that water-extracted compound of *A. muricata* had not caused neurodegeneration in the basal ganglia.

Table 7 Histopathological evaluation of male rats receiving *A. muricata* for 26 wk

Organ	Microscopic findings	Male						Female					
		Control	Dose (mg/kg/d)					Control	Dose (mg/kg/d)				
		DW	10	100	1,000	2,000	2,000-R†	DW	10	100	1,000	2,000	2,000–R†
		n = 11	n = 12	n = 12	n = 12	n = 12	n = 12	n = 12	n = 12	n = 11	n = 12	n = 12	n = 12
Brain	–	0	0	0	0	0	0	0	0	0	0	0	0
Trachea/Thyroid	–	0	0	0	0	0	0	0	0	0	0	0	0
Lung	–	0	0	0	0	0	0	0	0	0	0	0	1
Heart	–	0	0	0	0	0	0	0	0	0	0	0	0
Liver	Vacuolar degeneration	4	3	2	2	8	3	0	0	0	2	0	1
Pancreas	–	0	0	0	0	0	0	0	0	0	0	0	0
Spleen	–	0	0	0	0	0	0	0	0	0	0	0	0
Esophagus/ Stomach	–	0	0	0	0	0	0	0	0	0	0	0	0
Intestine	–	0	0	0	0	0	0	0	0	0	0	0	0
Kidney	–	0	0	0	0	0	0	0	0	0	0	0	0
Adrenal gland	–	0	0	0	0	0	0	0	0	0	0	0	0
Salivary/ Mammary gland	–	0	0	0	0	0	0	0	0	0	0	0	0
Testis/Ovary	–	0	0	0	0	0	0	0	0	0	0	0	0

DW = distilled water, R† = recovery group

The results illustrated that the aqueous leaf extract of *A. muricata* orally administered at doses of 10 mg/kg/d, 100 mg/kg/d and 1,000 mg/kg/d did not cause any toxic signs to rats during the experimental period (26 wk). However, clinical study should further investigate the evidence-based results of organ weight, liver and the number of platelets as the result of liver disorder and thrombocytopenia in the female rats treated with the extract at the dose of 2,000 mg/kg/d.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors express their gratitude to Mr Detmontree Wachisunthon for the HPLC analysis, the Herbarium Laboratory and Herbal Quality Assurance Center, Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Bangkok, Thailand, for plant identification, raw materials and extracts preparation as well as Professor Dr Thaweesak Songserm for examination of the histopathological slides.

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